

# Involvement of Plasma Membrane Redox Activity and Calcium Homeostasis in the UV-B and UV-A/Blue Light Induction of Gene Expression in Arabidopsis

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UV and blue light are important regulators of plant gene expression and development. We investigated the signal transduction processes involved in the induction of chalcone synthase (*CHS*) and phenylalanine ammonia-lyase (*PAL*) gene expression by UV-B and UV-A/blue light in an Arabidopsis cell suspension culture. Experiments with electron transport inhibitors indicated that plasma membrane redox activity is involved in both signal transduction pathways. Calcium ionophore treatment stimulated expression of the *TOUCH3* gene, and this induction was strongly antagonized by UV-A/blue and UV-B light, suggesting that both light qualities may promote calcium efflux from the cytosol. Consistent with this hypothesis, experiments with specific inhibitors indicated that UV-B and UV-A/blue light regulate calcium levels in a cytosolic pool in part via the action of specific  $\text{Ca}^{2+}$ -ATPases. On the basis of these and previous findings, we propose that plasma membrane redox activity, initiated by photoreception, is coupled to the regulation of calcium release from an intracellular store, generating a calcium signal that is required to induce *CHS* expression.

## INTRODUCTION

Light in the UV and blue regions of the spectrum has a major regulatory influence on plant growth and development (Short and Briggs, 1994; Jenkins et al., 1995). For instance, UV and blue light play key roles in phototropism, stomatal opening, cell extension, flowering time, and the expression of various genes. Recent research has identified several photoreceptors, distinct from the phytochromes, that mediate a range of responses to UV and blue light. In contrast, relatively little information is available on the signal transduction processes initiated by the photoreceptors, although the elucidation of these mechanisms is clearly essential to understanding UV and blue light responses. A powerful way to dissect these signaling pathways is the combined application of biochemical, cell physiological, molecular, and genetic approaches in Arabidopsis (Jenkins, 1997).

One of the photoreceptors that mediates responses to UV-A (320 to 390 nm) and blue light is cryptochrome1 (CRY1) (Ahmad and Cashmore, 1996). CRY1 is involved in controlling several photoresponses in Arabidopsis, including the suppression of hypocotyl and petiole extension, the promotion of cotyledon expansion, and the production of anthocyanin pigments (Koornneef et al., 1980; Ahmad et al., 1995; Jackson and Jenkins, 1995; Lin et al., 1995). CRY1

has sequence similarity to microbial DNA photolyases in the N-terminal region (Ahmad and Cashmore, 1993). Although CRY1 lacks photolyase activity, it binds similar flavin and pterin chromophores to the photolyases when expressed in heterologous systems (Lin et al., 1995; Malhotra et al., 1995). The DNA photolyases catalyze blue light-dependent repair of damaged nucleotides via an electron transfer mechanism (Sancar, 1994), raising the intriguing possibility that CRY1 may initiate UV-A/blue light signal transduction through a similar process. In support of this notion, there is evidence that various blue light responses involve plasma membrane redox processes, although none are related to a specific photoreceptor (Rubinstein and Luster, 1993; Asard et al., 1995).

The recently identified CRY2 protein resembles CRY1 in the chromophore binding region but differs in its C-terminal domain (Lin et al., 1998). CRY2 participates in controlling extension growth but has an additional function in the regulation of flowering (Guo et al., 1998). Recent evidence suggests that CRY1 and CRY2 are involved in phototropic responses in Arabidopsis (Ahmad et al., 1998), although there is strong evidence that the NONPHOTOTROPIC HYPOCOTYL1 protein is the photoreceptor for phototropism (Briggs and Liscum, 1997; Huala et al., 1997). Physiological and genetic studies indicate that additional UV and blue light receptors are present in plants, including a putative UV-B (280 to 320 nm) photoreceptor (Jenkins et al., 1997) and a photoreceptor for stomatal opening.

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UV and blue light regulate the expression of various plant genes. In several species, UV-B, UV-A, and blue light stimulate the transcription of genes encoding the key phenylpropanoid and flavonoid biosynthesis enzymes, phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) (Chappell and Hahlbrock, 1984; Kubasek et al., 1992; Jenkins, 1997; Schäfer et al., 1997). Studies of *cry1* (*hy4*) plants indicate that in Arabidopsis, the induction of *CHS* gene expression in UV-A/blue light is substantially mediated by CRY1 (Ahmad et al., 1995; Jackson and Jenkins, 1995), whereas a different photoreception system is responsible for induction in UV-B light (Fuglevand et al., 1996). Thus, at least two distinct phototransduction pathways induce *CHS* transcription in Arabidopsis. These pathways operate in mature Arabidopsis leaf tissue, whereas the phytochrome regulation of *CHS* is confined to seedlings <6 days old (Kaiser et al., 1995). Expression in UV-B is further increased by synergistic interactions with separate blue and UV-A signaling pathways that are not dependent on CRY1 (Fuglevand et al., 1996). It was shown recently that the induction of *CHS* transcription in Arabidopsis by UV-B and UV-A/blue light is mediated by identical regions of the promoter (Hartmann et al., 1998). Moreover, the "light-responsive unit" in the promoter that interacts with basic leucine zipper and MYB-related transcription factors is sufficient to confer a response to both light qualities.

By using a pharmacological approach in an Arabidopsis cell suspension culture, Christie and Jenkins (1996) identified some of the components of the inductive UV-A/blue (CRY1-mediated) and UV-B signaling pathways regulating *CHS* expression. Experiments with specific calcium channel blockers indicated that calcium efflux from an intracellular pool, rather than influx from outside the cell, was involved in both signaling pathways. However, only the UV-B pathway involved calmodulin. Calcium and calmodulin are also involved in the UV induction of *CHS* in parsley cells (Frohnmeier et al., 1997). In addition, protein kinase and phosphatase activities appear to be components of the UV-B and UV-A/blue phototransduction pathways, but the specific proteins have not been identified. Noh and Spalding (1998) reported that an anion channel blocker inhibited blue light-induced anthocyanin accumulation in Arabidopsis seedlings, but it had no effect on the accumulation of *PAL* and *CHS* transcripts. Thus, although some progress has been made, detailed information on the distinct UV-B and UV-A/blue light signaling pathways that regulate *CHS* expression in Arabidopsis is lacking. In particular, it is important to identify the initial events after photoreception, to establish how these events generate a calcium signal, to determine the nature of this signal, and to define the downstream processes that result in the stimulation of transcription.

In this study, we report the involvement of plasma membrane redox processes in the signal transduction pathways coupling UV-B and UV-A/blue light to *CHS* and *PAL* gene expression. In addition, we provide evidence that UV-B and UV-A/blue light regulate calcium levels in a cytosolic pool involved in gene expression in part via the action of specific

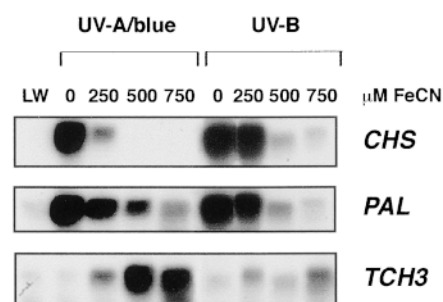
$\text{Ca}^{2+}$ -ATPases. On the basis of these and previous observations (Christie and Jenkins, 1996), we propose a model in which plasma membrane redox activity, initiated by photoreception, is coupled to the regulation of calcium release from an intracellular store, generating a calcium signal that is required to induce *CHS* expression.

## RESULTS

### The Electron Acceptor Ferricyanide Inhibits the UV-B and UV-A/Blue Light Induction of *CHS* and *PAL* Transcripts

We used an Arabidopsis cell suspension culture (May and Leaver, 1993; Christie and Jenkins, 1996) to investigate the signaling processes involved in the induction of *CHS* expression by UV-B and UV-A/blue light. The cells were grown routinely in a low fluence rate ( $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$ ) of white light; under these conditions, they contain very low levels of *CHS* transcripts, as shown in Figure 1 (first lane). Exposure of the cells to either UV-A/blue or UV-B light stimulates *CHS* transcript accumulation within a few hours (Christie and Jenkins, 1996), and *PAL* transcripts are induced similarly (J.M. Christie and G.I. Jenkins, unpublished data). The transcript levels after 6 hr of illumination are shown in Figure 1 (second and sixth lanes). In this system, there is no phytochrome induction (Christie and Jenkins, 1996). Hence, the cells respond similarly to mature Arabidopsis leaf tissue (Jackson et al., 1995; Fuglevand et al., 1996).

To test the hypothesis that UV-B and UV-A/blue light perception may initiate electron transport, we examined the ef-



**Figure 1.** Effect of Ferricyanide on *CHS*, *PAL*, and *TCH3* Expression in UV-A/Blue and UV-B Light.

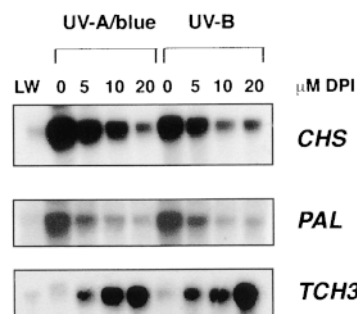
Cells grown in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light were transferred to either  $80 \mu\text{mol m}^{-2} \text{sec}^{-1}$  UV-A/blue or  $3 \mu\text{mol m}^{-2} \text{sec}^{-1}$  UV-B light for 6 hr in the presence of potassium ferricyanide (FeCN) at the concentrations indicated. Control cells (LW) were not treated with ferricyanide and were kept in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light for 6 hr. Cells were harvested, and *CHS*, *PAL*, and *TCH3* transcript levels were measured by sequential hybridization of DNA probes to blots of total RNA.

fect of the artificial electron acceptor potassium ferricyanide on *CHS* and *PAL* expression. Ferricyanide does not enter intact cells and has been used widely as an external electron acceptor in studies of plasma membrane redox activity (Rubinstein and Luster, 1993). Ferricyanide has been shown to inhibit several blue light responses, including  $H^+$  excretion in guard cell protoplasts (Gautier et al., 1992) and photopolarization of *Fucus* zygotes (Berger and Brownlee, 1994). We added ferricyanide to the cells at the time of transfer to UV-B and UV-A/blue light and measured transcript levels after 6 hr of illumination. The electron acceptor inhibited *CHS* and *PAL* transcript accumulation under both light qualities (Figure 1). Inhibition was concentration dependent, and the effective concentration was similar to that in other systems (e.g., Gautier et al., 1992). Potassium ferrocyanide at the same concentrations had no inhibitory effect (data not shown), indicating that the inhibition by ferricyanide is due to its functioning as an electron acceptor. We hypothesize that the transfer of electrons to ferricyanide perturbs redox processes in the plasma membrane and "short circuits" the initiation of UV-B and UV-A/blue light signal transduction.

#### A Flavoprotein Antagonist Inhibits the UV-B and UV-A/Blue Light Induction of *CHS* and *PAL*

Membrane-associated electron transport systems often include flavoproteins. Moreover, CRY1 binds a flavin chromophore (Lin et al., 1995; Malhotra et al., 1995). Therefore, we investigated whether a flavoprotein is involved in the UV-B and UV-A/blue light induction of *CHS* and *PAL* expression by examining the effects of the well-established inhibitor diphenylene iodonium (DPI). This compound is a flavoprotein antagonist that acts by drawing an electron away from the reduced flavin of its target protein (O'Donnell et al., 1993). It has been shown to inhibit redox enzymes in plant plasma membranes, including NADPH oxidase (Levine et al., 1994) and an NAD(P)H:quinone oxidoreductase (Trost et al., 1997). We preincubated cells with DPI for 1 hr in non-inductive  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light before transfer to UV-B or UV-A/blue light. Figure 2 shows that DPI inhibited both the UV-B and UV-A/blue light induction of *CHS* and *PAL* transcripts. Inhibition was observed at concentrations similar to those used in previous studies. We conclude that the UV-B and UV-A/blue light induction of gene expression involves the activity of one or more flavoproteins. It should be noted that some other flavin antagonists (phenylacetic acid, salicylhydroxamic acid, and sodium azide) also inhibited *CHS* expression but appeared to have general deleterious effects on the cells (J.M. Christie and G.I. Jenkins, unpublished data).

It was important to demonstrate that DPI and ferricyanide do not inhibit gene expression through a general effect on transcription or transcript stability or via an effect on cell viability. Therefore, we examined their effects on a control response. We reported previously (Christie and Jenkins, 1996)



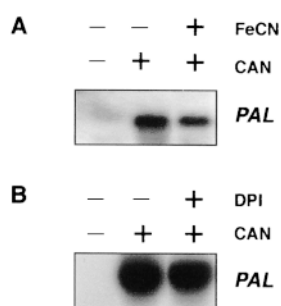
**Figure 2.** Effect of DPI on *CHS*, *PAL*, and *TCH3* Expression in UV-A/Blue and UV-B Light.

Cells grown in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light were incubated for 1 hr in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light with DPI at the concentrations indicated and then transferred to either  $80 \mu\text{mol m}^{-2} \text{sec}^{-1}$  UV-A/blue or  $3 \mu\text{mol m}^{-2} \text{sec}^{-1}$  UV-B light for 6 hr. Control cells (LW) were not treated with DPI and were kept in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light for 6 hr. Cells were harvested, and *CHS*, *PAL*, and *TCH3* transcript levels were measured by sequential hybridization of DNA probes to blots of total RNA.

that the addition of the protein phosphatase inhibitor cantharidin to the Arabidopsis cells stimulated *PAL* transcript accumulation. Cantharidin activates a defense signaling pathway (Levine et al., 1994; MacKintosh et al., 1994; Mathieu et al., 1996). Hence, the cantharidin induction of *PAL* provides a convenient control response to examine the effects of compounds that inhibit the light induction of *CHS* and *PAL*. As shown in Figures 3A and 3B, we found that DPI and ferricyanide had little effect on this control response at concentrations that strongly inhibited the UV-B and UV-A/blue light induction of *CHS* and *PAL* expression. We conclude that DPI and ferricyanide do not have general inhibitory effects on cell viability or gene expression.

#### Ferricyanide and DPI Stimulate Expression of the *TOUCH3* Gene

As a further control, we examined the effects of DPI and ferricyanide on another gene expression response. We reported previously that raising the cytosolic calcium concentration ( $[Ca^{2+}]_{\text{cyt}}$ ) in Arabidopsis cells with the ionophore A23187 in the presence of 10 mM external calcium induced expression of the Arabidopsis *TOUCH3* (*TCH3*) gene in noninductive  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light (Christie and Jenkins, 1996). *TCH3* encodes a calmodulin-like protein, and its expression is stimulated by a range of treatments that raise  $[Ca^{2+}]_{\text{cyt}}$ , including mechanical stimulation and low temperature (Braam and Davis, 1990; Braam et al., 1997). In fact, *TCH3* expression provides a sensitive physiological reporter of the  $[Ca^{2+}]_{\text{cyt}}$  (Braam, 1992; Braam et al., 1997; M.R. Knight, personal communication). Surprisingly, we found



**Figure 3.** Effect of DPI and Ferricyanide on the Induction of *PAL* by Cantharidin.

**(A)** Cells grown in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light were incubated in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light either with (+) or without (–)  $750 \mu\text{M}$  ferricyanide (FeCN) in the presence (+) or absence (–) of  $200 \mu\text{M}$  cantharidin (CAN). The cells were harvested after 6 hr, and *PAL* transcript levels were measured by hybridization of a DNA probe to a blot of total RNA.

**(B)** Cells grown in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light were incubated for 1 hr in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light either with (+) or without (–)  $20 \mu\text{M}$  DPI. A  $200\text{-}\mu\text{M}$  concentration of cantharidin was then added (+), or not added (–), to the cells. The cells were harvested after 6 hr, and *PAL* transcript levels were measured as described above.

that ferricyanide and DPI both induced a substantial increase in *TCH3* transcript accumulation without the addition of the ionophore and  $\text{Ca}^{2+}$ .

The stimulation of *TCH3* expression paralleled the inhibition of *CHS* and *PAL* expression in UV-B and UV-A/blue light, as shown in Figures 1 and 2, but was also observed in noninductive  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light (data not shown). In this white light treatment, the stimulation of *TCH3* transcript accumulation was transient, being maximal  $\sim 2$  hr after the addition of ferricyanide or DPI. These results provide further evidence that ferricyanide and DPI do not generally inhibit gene expression. In addition, they indicate that ferricyanide and DPI elevate  $[\text{Ca}^{2+}]_{\text{cyt}}$ , similar to the effect of the calcium ionophore.

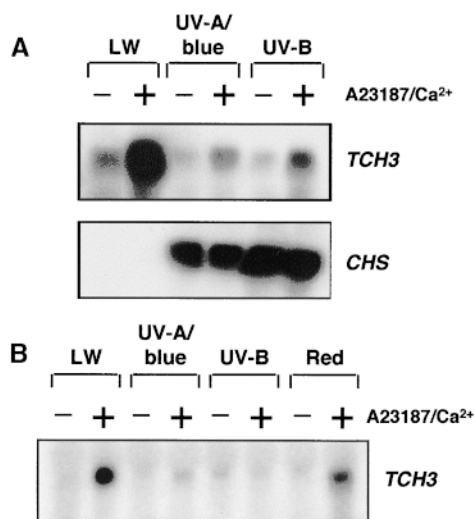
Hence, in our study, it was important to establish whether ferricyanide and DPI inhibited the UV-B and UV-A/blue light induction of *CHS* and *PAL* expression through an effect on electron transport per se or via the elevation of  $[\text{Ca}^{2+}]_{\text{cyt}}$ . As shown in Figure 4A (second lane), artificially elevating cytosolic calcium by treatment with A23187 and  $\text{Ca}^{2+}$  stimulated *TCH3* transcript accumulation in a noninductive, low fluence rate of white light, as reported previously (Christie and Jenkins, 1996). We used the ionophore to examine the effects of raising  $[\text{Ca}^{2+}]_{\text{cyt}}$  on *CHS* expression. As shown in Figure 4A (fourth and sixth lanes), the ionophore and  $\text{Ca}^{2+}$  treatment did not inhibit *CHS* expression (*PAL* expression; data not shown) in either UV-B or UV-A/blue light. Therefore, we conclude that the inhibition of *CHS* and *PAL* expression by ferri-

cyanide and DPI occurs through a direct effect on electron transport and not via an increase in  $[\text{Ca}^{2+}]_{\text{cyt}}$ .

### UV-B and UV-A/Blue Light Antagonize the Increase in *TCH3* Expression Mediated by Cytosolic Calcium

As a control in the experiment described above, we examined the effects of UV-B and UV-A/blue light treatments on the induction of *TCH3*. Unexpectedly, we observed that the stimulation of *TCH3* expression by A23187 and  $\text{Ca}^{2+}$  was much reduced in the presence of either UV-B or UV-A/blue light (Figures 4A and 4B). Note that the autoradiograph at the top in Figure 4A is overexposed to reveal the level of *TCH3* transcripts in UV-A/blue and UV-B light. Clearly, the level of *TCH3* expression under these lighting conditions in the presence of the ionophore (fourth and sixth lanes) is similar to that in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light without the ionophore (first lane).

We investigated the wavelength specificity of the inhibition of *TCH3* expression and, as shown in Figure 4B, found



**Figure 4.** Effect of the Elevation of  $[\text{Ca}^{2+}]_{\text{cyt}}$  on *CHS* Expression and of Light Treatments on Ionophore-Induced *TCH3* Expression.

**(A)** Cells grown in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light were incubated for 1 hr in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light either with (+) or without (–)  $10 \mu\text{M}$  A23187 and  $10 \text{ mM}$   $\text{Ca}^{2+}$ . Cells were then either kept in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light for 6 hr (LW) or transferred to  $80 \mu\text{mol m}^{-2} \text{sec}^{-1}$  UV-A/blue or  $3 \mu\text{mol m}^{-2} \text{sec}^{-1}$  UV-B light for 6 hr. Cells were harvested, and *CHS* and *TCH3* transcript levels were measured by sequential hybridization of DNA probes to blots of total RNA. The autoradiograph at top is overexposed to reveal the low level of *TCH3* transcripts in some treatments.

**(B)** Cells were treated as given in (A), and additional samples were transferred to  $80 \mu\text{mol m}^{-2} \text{sec}^{-1}$  red light for 6 hr.

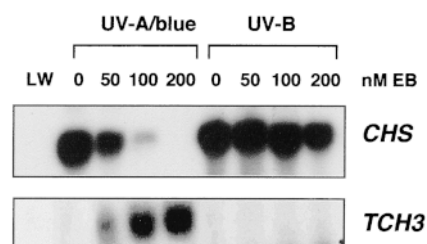
that red light had no effect. These results suggest that illumination specifically with UV-B and UV-A/blue light lowered the  $[Ca^{2+}]_{cyt}$  established by the ionophore treatment and hence reduced the level of *TCH3* expression. In fact, in the absence of the ionophore, the level of *TCH3* transcripts is lower in UV-A/blue and UV-B light than in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light (Figure 4A; cf. the third and fifth lanes with the first lane), further indicating that these light qualities lower  $[Ca^{2+}]_{cyt}$ . These observations lead us to hypothesize that UV-B and UV-A/blue light can, under certain conditions, stimulate mechanisms that result in a net efflux of calcium out of the cytosol.

### Involvement of $Ca^{2+}$ -ATPases in UV-B and UV-A/Blue Light Signal Transduction

$Ca^{2+}$ -ATPases provide an important means of calcium flux out of the cytosol into internal cellular compartments or the apoplast. These enzymes have been identified in the plasma membrane and internal membrane systems of several species (Bush, 1995; Askerlund and Sommarin, 1996). Hence, if UV-B and UV-A/blue light stimulate a net calcium flux out of the cytosol, then  $Ca^{2+}$ -ATPases are likely to be involved. Therefore, we investigated whether specific inhibitors of  $Ca^{2+}$ -ATPases affected the UV-B and UV-A/blue light induction of *CHS* expression. Some compounds (including thapsigargin) had no effect in this system, whereas others (including cyclopiazonic acid and 2,5-di[*tert*-butyl]-1,4-benzohydroquinone) inhibited expression in both light qualities (data not shown).

A particularly interesting result was obtained with erythrosin B (EB), which in the nanomolar range specifically inhibits  $Ca^{2+}$ -ATPases (Williams et al., 1990; Bush, 1995; Askerlund and Sommarin, 1996). As can be seen in Figure 5, preincubation of cells for 1 hr with 100 nM EB strongly inhibited the induction of *CHS* expression in UV-A/blue light but had no effect in UV-B. Similar results were obtained with the related compound eosin Y (data not shown). If EB inhibited the activity of a  $Ca^{2+}$ -ATPase specifically in UV-A/blue light, it would be expected to elevate the  $[Ca^{2+}]_{cyt}$  and stimulate *TCH3* expression. This is what we observed (Figure 5). In contrast, *TCH3* expression was not elevated by EB in UV-B light.

We previously reported that the calmodulin antagonist W-7 inhibited *CHS* expression in UV-B but not UV-A/blue light, which is the opposite of the effect of EB (Christie and Jenkins, 1996). Because some  $Ca^{2+}$ -ATPases are activated by calmodulin (Bush, 1995; Askerlund and Sommarin, 1996), we considered that the effect of W-7 in this system could be on a calmodulin-stimulated  $Ca^{2+}$ -ATPase involved in UV-B signal transduction. If this were the case, W-7 would be expected to inhibit efflux from the cytosol specifically in UV-B light. The results presented in Figure 6 are consistent with this hypothesis. The ionophore induced *TCH3* expression in low-fluence-rate white light (second lane), but UV-B inhib-



**Figure 5.** Effect of EB on *CHS* and *TCH3* Expression in UV-A/Blue and UV-B Light.

Cells grown in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light were incubated for 1 hr in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light with EB at the concentrations indicated and then transferred to either  $80 \mu\text{mol m}^{-2} \text{sec}^{-1}$  UV-A/blue or  $3 \mu\text{mol m}^{-2} \text{sec}^{-1}$  UV-B light for 6 hr. Control cells (LW) were not treated with EB and were kept in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light for 6 hr. Cells were harvested, and *CHS* and *TCH3* transcript levels were measured by sequential hybridization of DNA probes to blots of total RNA.

ited the induction (cf. the second and seventh lanes), as shown above (Figure 4). Preincubation of cells with W-7 overcame the inhibition of ionophore-induced *TCH3* expression by UV-B light (Figure 6; cf. the seventh and eighth lanes). In contrast, W-7 did not have an effect on the inhibition of *TCH3* expression by UV-A/blue light (cf. fifth and eighth lanes).

The data in Figures 5 and 6 therefore suggest that inhibition of particular  $Ca^{2+}$ -ATPases in UV-B and UV-A/blue light reduces the efflux of calcium from the cytosol, causing an increase in  $[Ca^{2+}]_{cyt}$  and a consequent stimulation of *TCH3* expression. Moreover, inhibition of the putative  $Ca^{2+}$ -ATPases in the different light qualities is accompanied by the inhibition of *CHS* expression.

## DISCUSSION

### Plasma Membrane Redox Processes Are Involved in CRY1 and UV-B Signal Transduction Regulating *CHS* and *PAL*

The fact that both the electron acceptor ferricyanide and the flavoprotein antagonist DPI inhibited the induction of *CHS* and *PAL* gene expression by UV-B and UV-A/blue light indicates that redox processes are involved in these signaling pathways. It is clear that the effect of ferricyanide was related specifically to its action as an electron acceptor because ferrocyanide had no effect. Inhibition was not the result of general deleterious effects on gene expression or cell viability because ferricyanide and DPI actually stimulated expression of the *TCH3* gene. Moreover, these compounds

had little effect on the induction of *PAL* expression by the protein phosphatase inhibitor cantharidin. There are a number of other reports of redox processes being involved in blue light responses (e.g., Dharmawardhane et al., 1989; Raghavendra, 1990; Gautier et al., 1992; Rubinstein and Luster, 1993; Berger and Brownlee, 1994; Asard et al., 1995), and our results are entirely consistent with these findings. Thus, it appears that electron transport may be a general component of blue light signal transduction.

Our hypothesis is that CRY1 and the UV-B photoperception system both initiate electron transport in the plasma membrane. The diversion of electrons externally to the cell-impermeant acceptor ferricyanide may prevent their transfer to endogenous electron acceptors that are involved in signal transduction. Moreover, this disruptive effect of ferricyanide on redox activity may affect the membrane potential and ion fluxes established as a result of photoreception that are required for signal transduction. The involvement of electron transport is not surprising given that CRY1 is a flavoprotein (Lin et al., 1995; Malhotra et al., 1995) and that electron transfer is the primary mechanism of action of the related DNA photolyases (Sancar, 1994). Although CRY1 is a soluble protein (Lin et al., 1996), it could interact with the plasma membrane to initiate electron transport and signal transduction, perhaps via the C-terminal domain. This domain is essential for function and could mediate protein-protein interactions (Ahmad et al., 1995).

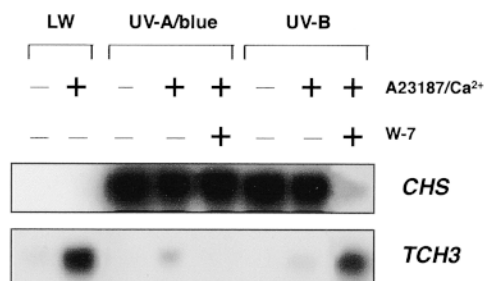
The mechanism of UV-B light detection for *CHS* and *PAL* induction remains unknown, but our results indicate that the perception of UV-B initiates redox processes in the plasma membrane. It is conceivable that UV-B is detected by an un-

characterized photoreceptor with flavin and/or pterin chromophores (Jenkins et al., 1997). On the other hand, at least some UV-B responses are mediated by the generation of reactive oxygen species (ROS) (Green and Fluhr, 1995), and this might involve a plasma membrane redox system similar to that implicated in defense gene activation (Jabs et al., 1997). However, our studies (J.C. Long and G.I. Jenkins, unpublished data) indicate that *CHS* expression in the Arabidopsis cells is not stimulated by ROS and that the UV-B induction of *CHS* is not prevented by ROS scavengers, so it appears that UV-B can regulate gene expression by different signaling pathways.

Although CRY1 is a flavoprotein, there is no evidence that DPI directly antagonizes either CRY1 or a putative UV-B photoreceptor. It could inhibit the action of a downstream electron transport component in the plasma membrane. It is reported that DPI inhibits an NADPH oxidase associated with the plasma membrane that is involved in defense signaling (Levine et al., 1994; Jabs et al., 1997; Lamb and Dixon, 1997). Moreover, NADPH oxidase activity is reported to increase in Arabidopsis plants exposed to UV-B (Rao et al., 1996). However, because the mechanism of action of DPI indicates that it may antagonize the action of various flavoproteins (O'Donnell et al., 1993), there is no direct evidence that NADPH oxidase is the target of DPI in our experiments.

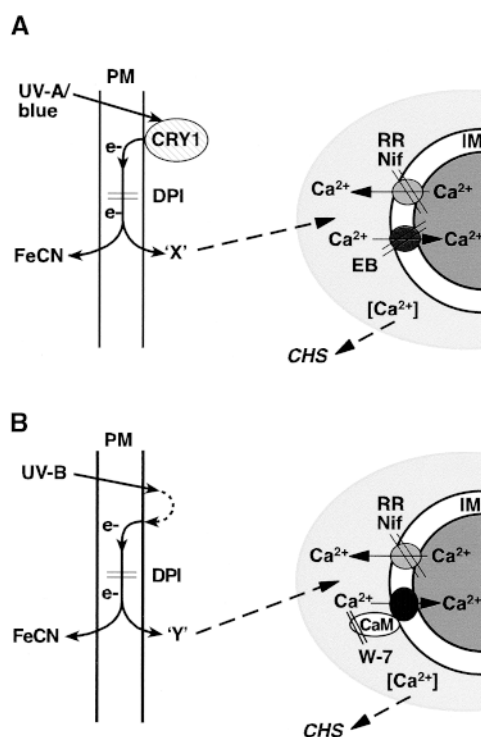
We conclude that the plasma membrane is the site of early events in phototransduction coupled to *CHS* and *PAL* expression, as indicated in the model presented in Figures 7A and 7B. We do not know the identity of the end products of redox activity, shown as 'X' and 'Y' in Figure 7. It is possible that redox activity results in the activation of one or more specific signaling components, for example, by phosphorylation, that produce a diffusible second messenger. Alternatively, electron transport may simply generate ion fluxes required for signaling. Our findings are not unprecedented because there is increasing evidence in animal systems for the involvement of plasma membrane redox activity in several signal transduction pathways regulating gene expression (Medina et al., 1997).

Our data indicate that the inhibition of plasma membrane redox processes by ferricyanide and DPI results in an elevation of  $[Ca^{2+}]_{cyt}$ , because *TCH3* transcripts increase in the absence of the ionophore and  $Ca^{2+}$  treatment. *TCH3* expression is regulated by a range of treatments that elevate cytosolic calcium and is a sensitive indicator of the  $[Ca^{2+}]_{cyt}$  (Braam, 1992; Braam et al., 1997; M.R. Knight, personal communication). Although the inhibition of *CHS* and *PAL* expression in UV-B and UV-A/blue light correlates with an increase in *TCH3* expression, a transient increase in *TCH3* transcripts was observed in noninductive white light in the presence of ferricyanide and DPI. Thus, it appears that the elevation of  $[Ca^{2+}]_{cyt}$  is not specifically a result of the inhibition of UV-B and UV-A/blue light signal transduction. As shown in Figure 4A, this increase in  $[Ca^{2+}]_{cyt}$  is not the cause of the inhibition of *CHS* and *PAL* expression.



**Figure 6.** W-7 Overcomes the Inhibitory Effect of UV-B on *TCH3* Expression.

Cells grown in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light were incubated for 1 hr in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light either with (+) or without (-)  $100 \mu\text{M}$  W-7. A  $10\text{-}\mu\text{mol m}^{-2} \text{sec}^{-1}$  concentration of A23187 and  $10 \text{ mM}$   $Ca^{2+}$  were added (+) or not added (-), and the cells were incubated for an additional hour. The cells were then either kept in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light (LW) or transferred to  $80 \mu\text{mol m}^{-2} \text{sec}^{-1}$  UV-A/blue or  $3 \mu\text{mol m}^{-2} \text{sec}^{-1}$  UV-B light for 6 hr. Cells were harvested, and *CHS* and *TCH3* transcript levels were measured by sequential hybridization of DNA probes to blots of total RNA.



**Figure 7.** Model of the UV-A/Blue and UV-B Light Signaling Pathways Regulating *CHS* Expression.

**(A)** UV-A/blue light signaling.

**(B)** UV-B light signaling.

The perception of UV-A/blue light by CRY1 in **(A)** and of UV-B light by an unknown process in **(B)** is proposed to initiate electron ( $e^-$ ) transport in the plasma membrane (PM), which is inhibited by DPI or ferricyanide (FeCN). 'X' in **(A)** and 'Y' in **(B)** refer to hypothetical ion fluxes or diffusible second messengers generated by electron transport that are proposed to regulate the release of calcium from an internal store (dark shading), which is bound by an internal membrane (IM), into an associated cytosolic pool (light shading). The  $[Ca^{2+}]$  in this cytosolic pool is a necessary component of the signal that induces *CHS* expression. Calcium release from the internal store is proposed to be via nifedipine (Nif)- and ruthenium red (RR)-sensitive channels. Calcium uptake into the internal store is hypothesized to involve either an EB-sensitive  $Ca^{2+}$ -ATPase (CRY1 mediated pathway in **(A)**) or a calmodulin (CaM)-stimulated  $Ca^{2+}$ -ATPase inhibited by W-7 (UV-B pathway in **(B)**).

### Calcium Homeostasis in CRY1 and UV-B Signal Transduction

We reported previously that the UV-B and UV-A/blue light induction of *CHS* expression involved calcium because the responses were inhibited by the calcium channel blockers nifedipine and ruthenium red (Christie and Jenkins, 1996). Significantly, lanthanum had no effect on induction, indicat-

ing that an intracellular calcium pool, rather than an influx of extracellular calcium, was involved in the responses. However, raising cytosolic calcium artificially by A23187 and  $Ca^{2+}$  treatment was not sufficient to stimulate *CHS* expression. Therefore, we proposed that UV-B and UV-A/blue light elevate cytosolic calcium in a specific intracellular pool or microdomain that is not readily accessed by the cytosolic calcium raised by ionophore treatment. An alternative scenario is that UV-B and UV-A/blue light elevate a general cytosolic calcium pool but that an additional process(es) is required to stimulate *CHS* expression.

Our current data raise the possibility that plasma membrane electron transport may be required to stimulate *CHS* expression in conjunction with the intracellular calcium flux. However, it is unlikely that the photoreceptors would separately initiate electron transport at the plasma membrane and a calcium flux at an intracellular membrane. It is more likely that the two processes are coupled in a specific signal transduction pathway as the result of a single primary action of the photoreceptors. We propose that photoreception initiates electron transport at the plasma membrane and that consequent changes in ion fluxes, or the generation of a diffusible second messenger, stimulate a calcium flux from an intracellular store into an adjacent cytosolic pool (Figure 7). Similar processes have been described in other systems, including calcium-induced calcium release from intracellular stores and release promoted by inositol 1,4,5-triphosphate or cyclic ADP-ribose (Allen et al., 1995; Trewavas and Malhó, 1997; Sanders et al., 1998).

The experiments reported here provide evidence for an additional role of UV-B and UV-A/blue light in regulating cellular calcium in that they may, under certain conditions, stimulate calcium efflux from the cytosol. Both light qualities strongly antagonized the increase in *TCH3* expression induced by the ionophore and  $Ca^{2+}$  treatment. This effect was not mediated by red light, which would be detected by phytochrome. Whether this lack of effectiveness of phytochrome is restricted to the Arabidopsis cell suspension is not known.

Although it is conceivable that the UV-B and CRY1 signaling pathways inhibited *TCH3* expression by acting downstream of the cytosolic calcium pool, the experiments with EB and W-7 argue against this interpretation. The inhibition of *CHS* expression by the  $Ca^{2+}$ -ATPase inhibitor EB specifically in UV-A/blue light was coupled to the stimulation of *TCH3* expression. Similarly, W-7 specifically inhibits the UV-B induction of *CHS* (Christie and Jenkins, 1996) and, as shown in Figure 6, overcomes the inhibition of ionophore-stimulated *TCH3* expression by UV-B but not UV-A/blue light. W-7 would inhibit calmodulin-dependent  $Ca^{2+}$ -ATPases, and such an effect would explain these data. The effect of W-7 appears to be quite specific in this system; otherwise, it would have inhibited the UV-A/blue light response.

Thus, our results suggest that different types of  $Ca^{2+}$ -ATPase are associated with the UV-B and UV-A/blue light signaling pathways. Inhibition of either  $Ca^{2+}$ -ATPase appears

to raise  $[Ca^{2+}]_{cyt}$  and stimulate *TCH3* expression. However, as shown in Figure 4A, the general elevation of  $[Ca^{2+}]_{cyt}$  does not prevent *CHS* expression and therefore would not account for the inhibition of *CHS* expression by EB and W-7. Two possible explanations of this apparent paradox come to mind. One is that the calcium pool elevated by ionophore treatment is distinct from that raised by inhibition of the  $Ca^{2+}$ -ATPases and that an increase in  $[Ca^{2+}]$  above an optimal level in the latter pool may inhibit *CHS* expression. A second possible explanation is that the  $Ca^{2+}$ -ATPases replenish the internal stores from which UV-B and UV-A/blue light mobilize calcium. These stores may become significantly depleted in the light by calcium channel activity when  $Ca^{2+}$ -ATPase activity is inhibited by EB or W-7. This may limit the availability of calcium for the UV-B and UV-A/blue light induction of *CHS*.

Further research is needed to examine these possibilities. In particular, it is necessary to identify the  $Ca^{2+}$ -ATPases associated with UV-B and UV-A/blue light signaling and to determine their cellular location. Both calmodulin-dependent and calmodulin-independent  $Ca^{2+}$ -ATPases are reported to be present on intracellular membranes (Bush, 1995; Askerlund and Sommarin, 1996).

The potential significance of the regulation of  $Ca^{2+}$ -ATPases in plant signal transduction has been elegantly demonstrated in recent research. A  $Ca^{2+}$ -ATPase whose expression is induced by gibberellin in rice aleurone is reported to have a key role, in conjunction with calcium channels, in regulating a localized release of calcium from internal stores (Chen et al., 1997). In our proposed model (Figure 7), UV-B and CRY1 photoreception leads to an increase in  $[Ca^{2+}]$  in a cytosolic pool associated with an intracellular calcium store through the combined action of specific  $Ca^{2+}$ -ATPases and nifedipine- and ruthenium red-sensitive calcium channels. It appears that this homeostatic mechanism produces a calcium signal with the necessary spatial and temporal characteristics to initiate *CHS* and *PAL* expression. Although we have no direct evidence that the putative  $Ca^{2+}$ -ATPases are located on internal membranes or on the same membranes as the proposed calcium channels, the model presented is consistent with our findings. We believe the model is valuable because it provides a working hypothesis that can be tested.

One way in which we can test our hypothesis is by direct measurements of calcium fluxes. In contrast to a recent report (Lewis et al., 1997), we have observed that blue light induces an increase in  $[Ca^{2+}]_{cyt}$  in *Arabidopsis* seedlings (G. Baum, J.C. Long, G.I. Jenkins, and A.J. Trewavas, unpublished data). However, this stimulation is retained in a *cry1* null mutant and is inhibited by lanthanum, indicating that it is unrelated to the blue light stimulation of *CHS* expression. Currently, we do not know which tissues contribute to this calcium signal. Hence, to detect the calcium fluxes involved in the UV-B and UV-A/blue light induction of *CHS*, we need to focus the measurements on the specific cells that show the increase in expression.

## METHODS

### Plant Material and Experimental Treatments

The *Arabidopsis thaliana* cell suspension culture was grown photo-mixotrophically in continuous  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light with constant shaking, as described by Christie and Jenkins (1996). Cells were subcultured weekly and used for gene expression experiments on the third day after subculture. Ten-milliliter aliquots of cells were transferred aseptically to 50-mL flat tissue culture flasks. Compounds were added to the flasks as indicated, and the cells were either kept in noninductive  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light for 1 hr before inductive light treatment or illuminated immediately. Illuminations with  $3 \mu\text{mol m}^{-2} \text{sec}^{-1}$  UV-B,  $80 \mu\text{mol m}^{-2} \text{sec}^{-1}$  UV-A/blue, or  $80 \mu\text{mol m}^{-2} \text{sec}^{-1}$  red light were at  $21^{\circ}\text{C}$  for 6 hr, using the light sources described previously (Christie and Jenkins, 1996). Photon fluence rates were measured at the surface of the cells, taking into account light absorption by the flasks. Control cells were kept in  $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$  white light for the duration of the experiment. Throughout the treatments, cells were shaken at 80 rpm.

Stock solutions of potassium ferricyanide and erythrosin B (EB) were mixed in water, and those of diphenylene iodonium (DPI), W-7, and A23187 were mixed in DMSO. The volume added to a 10-mL aliquot of cells did not exceed  $100 \mu\text{L}$ , and the equivalent concentration of solvent alone had no effect on gene expression.

### Measurement of Transcript Levels

After illumination, cells were harvested into liquid nitrogen, and RNA was isolated as described previously (Jackson et al., 1995; Christie and Jenkins, 1996). Equal amounts of RNA (normally  $20 \mu\text{g}$ ) were applied to lanes of a 1.3% agarose-formaldehyde gel. Equal loading was confirmed by staining with ethidium bromide or hybridization to the constitutive *H1* DNA probe (Christie and Jenkins, 1996; data not shown). After electrophoresis, RNA was blotted onto nylon, and the blots were hybridized to the radiolabeled chalcone synthase (*CHS*), phenylalanine ammonia-lyase (*PAL*), or *TOUCH3* (*TCH3*) DNA probes (Jackson et al., 1995; Christie and Jenkins, 1996). Filters were washed under stringent conditions and autoradiographed. Radioactivity was removed from the filters before rehybridization with another probe. All experiments were repeated at least three times, and the data presented are representative of the results obtained.

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